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Full Length Research Paper

Purification and characterization of protease enzyme from *Burkholderia stabilis*

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***Burkholderia stabilis*, a protease producing organism was isolated from green house soils in Chungchugnam-do province, Gongju-Gun area in South Korea. Optimum protease activity of 116.4 U/ml was observed in the growth medium containing 0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, 0.05% citric acid dehydrate, 0.1% yeast extract and 0.2% casein. The protease production was found to be optimized in 1: 5 cultivation volume with 1% inoculum, shaken at 150 rpm. The enzyme was active in pH range 5 to11 and temperature of 30 to 80°C. The optimum pH and the temperature for protease activity were recorded to be pH 8 and 50°C, respectively. The enzyme was stable up to 40°C and pH 9. The protease activity was inhibited by Zn^{2+}, Ni^{2+} and Sn^{2+} and increased by Ca^{2+}, Mg^{2+} and Mn^{2+}. The maximum enzyme activity was displayed with casein as the substrate followed by egg albumin, gelatin and bovine serum albumin (BSA). V_{max} and K_m values were 89.28 U/ml and 0.82 mg/ml, respectively when casein was a substrate. The protease was purified to homogeneity by a combination of ammonium sulphate precipitation and gel filtration chromatography. The molecular weight of the enzyme was recorded as 45 kDa by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis.**

Key words: Purification, characterization, protease**,** *Burkholderia stabilis.*

INTRODUCTION

Proteases are one of the most important hydrolytic enzymes and they cleave peptide bonds of protein resulting into small peptides and free amino acids, which can be absorbed and utilized by living cells. Due to their wide spread substrate specificity, these proteases are extensively used for protein degradation in various Industrial processes particularly food, leather, textile, dairy, pharmaceutical and chemical industrial process. This accounts approximately 60% of the total industrial enzyme market (Rai and Mukherjee, 2010).

Though, widespread sources of proteases are often reported, the industrial demand is not yet sufficiently met (Karbalaei-Heidari et al., 2007). In this context, microbial proteases have gained substantial recognition as a preferred source of proteases, which can potentially meet the current demand for commercial proteases. This is apparently because of the physiological and biochemical properties and low production cost incurred with microbial proteases. Furthermore, microbes can genetically be manipulated to generate new enzymes for various applications. Proteases are complex enzymes that differ from each other in properties such as substrate specificity, active site and mechanism of action. For a successful enzyme production system, it is therefore needed to understand favorable culture conditions with optimum pH, temperature and media composition. Several factors including microorganism strain, production system, purification process, temperature, pH and substrate can affect the stability of microbial proteases in industrial processes, (Stoner et al., 2004). Most of the industrial processes are carried out under specific physical and chemical conditions, where many enzymes are often

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Abbreviations: BSA, Bovine serum albumin; **PMSF,** phenymethylsulfonyl fluoride; **EDTA,** tetra-sodium ethylene-diamine tetra acetate; **DTNB,** dithionitrobenzoic acid; **β-ME,** β-mercaptoethanol; **SDS-PAGE,** sodium dodecyl sulphate polyacrylamide gel electrophoresis.

unstable. Therefore, having available enzyme, which can resist harsh conditions in the industrial process is considered to be a great advantage.

A variety of microorganisms has been investigated in order to obtain new isolates which can ensure higher productivity and enzyme stability. The search for new species is continually stimulated by ever-increasing demand made by the protease-producing companies. In the present study, a protease producing bacteria, *Burkholderia stabilis* was isolated and the optimal conditions affecting the activity of pure enzyme were assessed under laboratory conditions.

MATERIALS AND METHODS

Isolation of protease producing bacteria

Soil collected from Chungchugnam-do province, Gongju-Gun area in South Korea was used in isolating protease producing bacteria using skim milk agar plates containing 1% Bacto tryptone, 0.5% NaCl, 0.5% Bacto yeast extract, 1% skim milk, and 1.6% agar. Plates were incubated at 37°C for two days. Colonies forming clear halos (Figure 1), because of partial hydrolysis of milk casein, were recorded as protease producing organisms (Olajuyigbe and Ajele, 2008). Selected strains were further screened for production of protease using standard protease assay procedure as described by Cupp-Enyard (2008). Bacterial strain that exhibited the highest protease activity was selected for further studies (SAph-24).

16S rDNA gene sequencing and phylogenetic analysis of the isolated bacteria

The partial sequencing of 16S rRNA of the strain was done with the help of DNA sequencing service, SOLGENT, Daejeon, South Korea using universal primers, 27F and 1492R. The online program, basic local alignment search tool (BLAST) was used to find out the related sequences with known taxonomic information available at the databank of NCBI (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree (Figure 2) was constructed using CLUSTAL X program, which involved sequence alignment by neighbor joining method and maximum parsimony using the MEGA4 program. Grouping of sequences was based on confidence values obtained by bootstrap analysis of 1,000 replicates. Gaps were edited in the BioEdit program and evolutionary distances were calculated using Kimura two parameter model. Reference sequences were retrieved from GenBank under the accession numbers indicated in the trees.

Cultivation of microorganism and protease production

Isolated strain was cultured in synthetic medium- casein (SMC; 0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, 0.05% citric acid dehydrate, 0.1% yeast extract and 0.2% casein) and incubated for 36 h with continuous shaking at 30°C. A sterilized uninoculated medium was served as the control. Protease activity and cell growth of culture were monitored every 6 h. Optical density of culture supernatant was measured using ultraviolet (UV) spectrophotometer at 660 nm to obtain the cell growth. Sample of cell culture and control were taken into centrifugation tube and centrifuged for 10 min at 12,000 rpm 4°C. The clear supernatant was subjected to detection of protease activity as described by Cupp-Enyard (2008).

Assay of protease activity

Protease activity was assayed by sigma's non-specific protease assay method described by Cupp-Enyard (2008) with some modifications. Casein solution (0.65 %) was prepared in 50 mM Tris-HCl buffer pH 7.4 and used as substrate. The reaction mixture was made up of 5 ml of the casein substrate and 1 ml of the centrifuged culture supernatant. After 30 min incubation at 37°C, the reaction was terminated by adding 5 ml of 110 mM trichloroacetic acid (TCA). The tubes were allowed to stand for 30 min 37°C. Then, each test solutions were filtered and 1 ml of the above filtered solution was taken into another test tube. Each test tube was incubated for 30 min at 37°C after adding 5 ml of 0.5 M sodium carbonate and 1 ml of Folin's reagent and absorbance was measured at 660 nm using UV spectrophotometer. Concentration of tyrosine produced by cultures was measured with the help of standard graph of tyrosine obtained in the range of 10 to 100 µg/ml. One unit of protease activity was defined as the amount of the enzyme resulting in the release 1 µg/ml of tyrosine per minute under the assay conditions.

Assay of protein

Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as the standard. The specific activity was expressed as the enzyme activity per mg of protein.

Optimization of protease production

Effect of different media on protease production

Various media on protease production were studied by culturing the isolated strain in 250 ml erlenmeyer flasks containing 100 ml of the different liquid media such as; synthetic medium-casein (SMC) containing 0.7% KH_2PO_4, 0.2% K_2HPO_4, 0.01% $MgSO_4.7H_2O$, 0.05% citric acid dehydrate, 0.1% yeast extract and 0.2% casein; nutrient broth supplemented with 1% casein (NBC), starch-soybean meal (SS) medium containing 2% starch, 1% soybean meal; soybean meal-tryptone (ST) medium containing 1% soybean meal, 1% tryptone and nutrient broth (NB) containing 1.3% nutrient broth. The medium which showed the highest protease activity was selected for further protease purification studies.

Effect of culture conditions on protease activity

The effect of various culture conditions such as shaking speed (50 to 200 rpm), cultivation volume (100 to 350 ml in 500 ml capacity flask), inoculum size (1 to10%) on protease production were determined by incubating the isolated strain under the above different conditions. Protease activity was measured according to standard procedure as described earlier.

Effect of pH on activity and stability of protease

The optimum pH for maximum protease activity was determined by performing protease activity assay at different pH values ranging from 5 to 11. For that purpose, 0.65 % casein was prepared by the following buffers: 0.05 M citrate-phosphate buffer (pH 5 to 6), Tris-HCl buffer (pH 7 to 8), and glycine buffer (pH 9 to 11). Reaction mixtures were incubated at 37°C for 30 min and the activity of protease was measured as described above. The effect of pH on protease stability was determined by pre-incubating the enzyme without substrate at different pH values (5 to 11) using different buffers. The residual protease activity was determined according to the assay procedure.

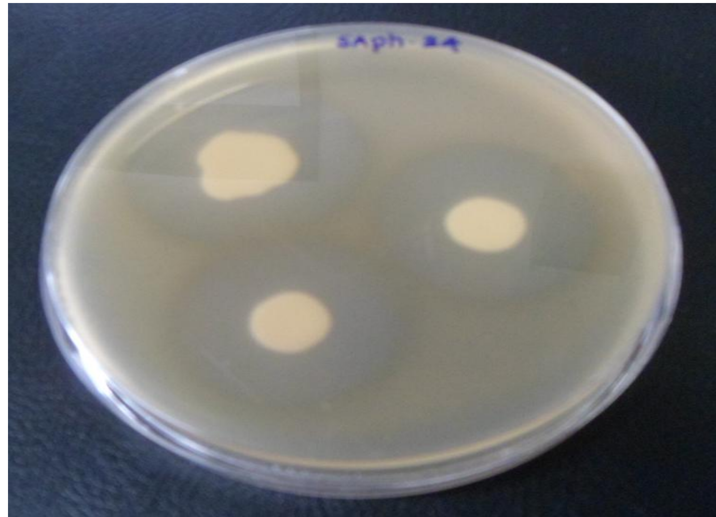

Figure 1. Halo zones produced by *Burkholderia stabilis* due to protease activity.

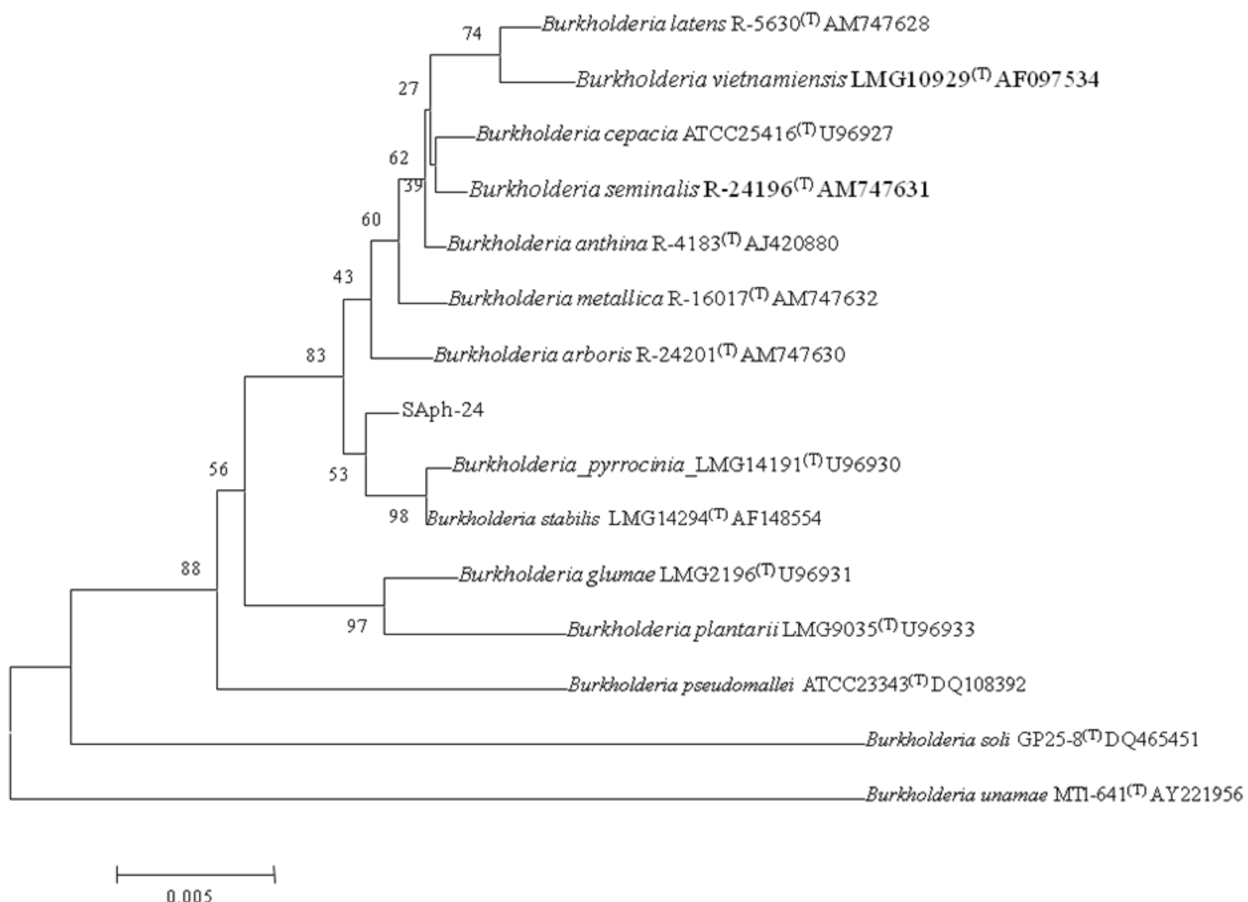

Figure 2. Phylogenetic tree based on 16S rDNA gene sequences, showing the position of strain SAph-24 with respect to related species. The scale bar indicates 0.005 substitutions per nucleotide position.

Effect of temperature on activity and stability of protease

The effect of temperature on the protease activity was determined by incubating the reaction mixtures at different temperatures ranging from 30 to 80°C. After incubation, protease activity was assayed. The effect of temperature on the protease stability was determined by pre-incubating the enzyme without substrate at different temperatures (30, 40, 50, 60, 70 and 80°C). The residual protease

activity was determined according to the assay procedure.

Effect of metal ions and protease inhibitors on protease activity

The effect of metal ions (Zn^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+}) on protease activity was tested by incubating enzyme solutions with each of the metal ions at a concentration of 1 and 10 mM prepared in 50 mM Tris-HCl buffer (pH 7.4). The remaining activity of the enzyme was measured under the standard procedure.

The effects of different inhibitors such as phenymethylsulfonyl fluoride (PMSF), tetra-sodium ethylene-diamine tetra acetate (EDTA), dithionitrobenzoic acid (DTNB), and β-mercaptoethanol (β-ME) on the activity of the protease were determined. The protease enzyme was pre-incubated at 37°C for 30 min with each inhibitor at the final concentration of 1 and 10 mM prepared in 50 mM Tris-HCl buffer (pH 7.4). The control was pre-incubated without any inhibitor and residual protease activity was measured.

Substrate specificity and enzyme kinetics

The substrate specificity of protease was studied by using different protein substrates such as casein, bovine serum albumin, egg albumin, and gelatin. Each substrate was dissolved in 50 mM Tris-HCl buffer (pH 7.4) to a concentration of 1 mg/ml and incubated with protease enzyme at 37°C for 30 min. Protease activity was determined same as the standard assay procedure. To determine the kinetic parameters, protease enzyme was incubated with various concentration of casein (1 to 20 mg/ml) prepared in 50 mM Tris-HCl buffer (pH 7.4). K_m and V_{max} were calculated by linear regression from Lineweaver-Burk plots (Lineweaver and Burk, 1934). All the experiments were conducted independently in triplicates.

Purification of protease

The organism was grown as described earlier and the cells were separated by centrifugation at 12,000 rpm, 4°C for 15 min after 24 h. The supernatant was used for the crude protease preparation. The enzyme was precipitated from the supernatant by the gradual addition of solid ammonium sulphate with gentle stirring to 50, 60 and 70% saturation and precipitate was collected by centrifugation at 20,000 rpm for 30 min. Then, the precipitate was dissolved in Tris-HCl buffer (50 mM, pH 7.4) and dialyzed overnight at 4°C against the same buffer. The protease activity and protein concentration were measured and specific activity was calculated.

Gel filtration chromatography

The dialyzed ammonium sulfate fractions were concentrated using an ultrafiltration (Amicon® Ultra-0.5) centrifugation filter devices. The resulting concentrated sample (4 to 5 ml, 2.0 to 2.5 mg) was applied to a Sephadex G-300 gel filtration column XK equilibrated with the Tris-HCl buffer (50 mM, pH 7.4). The protease was eluted at a rate of 1.5 ml/min. The fractions (approximately 1.5 ml of each) possessing protease activity were pooled and concentrated. The protein content was determined using the method previously described (Lowry et al., 1951), with BSA as the standard.

Molecular weight determination by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The purified, concentrated protease was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to estimate the molecular weight of the protein. SDS-PAGE was carried out according to the method of Laemmli (1970). However, the method was slightly modified with the use of 12% (w/v) separating gel and 5% (w/v) stacking gel. Concentrated samples were boiled for 3 to 4 min with sample loading dye before electrophoresis. Electrophoresis was carried out at a constant current of 100 V / 20 mA per gel until the tracking dye (bromophenol blue) reached the bottom of the gel (approximately 2 h total running time). Relative molecular weight of the enzyme was estimated by comparison with protein molecular weight markers (PageRuler™ Prestained Protein Ladder. Fermentas). After electrophoresis, the gel was stained with Coomassie brilliant blue to locate protein bands.

RESULTS AND DISCUSSION

Isolation and identification of protease producing microorganism

According to 16S rRNA sequence analysis, the isolated strain (SAph-24) using skim milk agar plates were identified as *B. stabilis.* Comparison of the 16S rRNA sequence among available strains of *Burkholderia* species showed high homology to *B. stabilis* LMG14294(T) AF148554 with 99.7% sequence identity*.* A phylogenetic tree was constructed with 16S rRNA sequence of strain with other *Burkholderia* species using neighbor-joining method (Figure 2).

Cultivation of microorganism and protease production

B. stabilis cultivated over a 36 h period showed that protease production increased exponentially after 6 h and reached to the peak after 24 h (116.6 U/ml) (Figure 3).

Effect of different media on protease production

The protease production was assessed with various media (SMC, NBC, SS, ST and NB). As shown in Figure 4, all the media were found to support protease production, though medium-based differences were revealed. The SMC medium was found to be the most suitable medium for the protease production as it produced significantly higher amount of protease (116.92 U/ml). This might be attributed to the availability of the medium components such as organic nitrogen source (yeast extract) and ions. Based on the results, SMC medium was selected for further optimization studies.

Effect of culture conditions on protease activity

A significantly higher (2.24 fold) protease activity was observed under 150 rpm shaking condition (136.8 ± 4.34

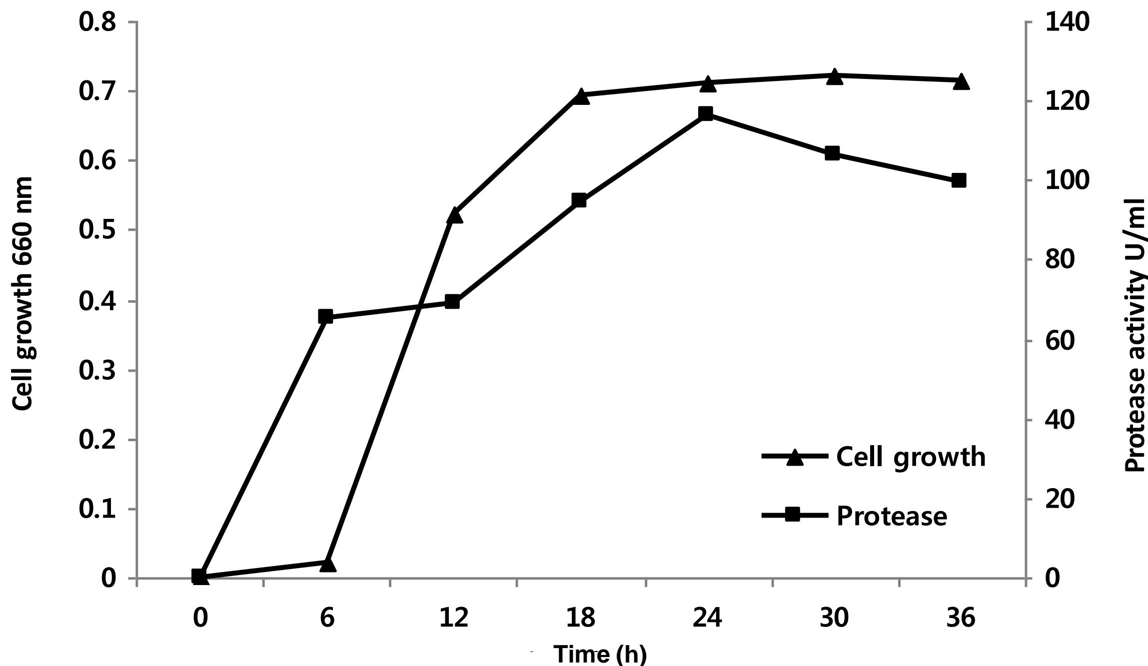

Figure 3. Growth kinetics and protease production of *B. stabilis.*

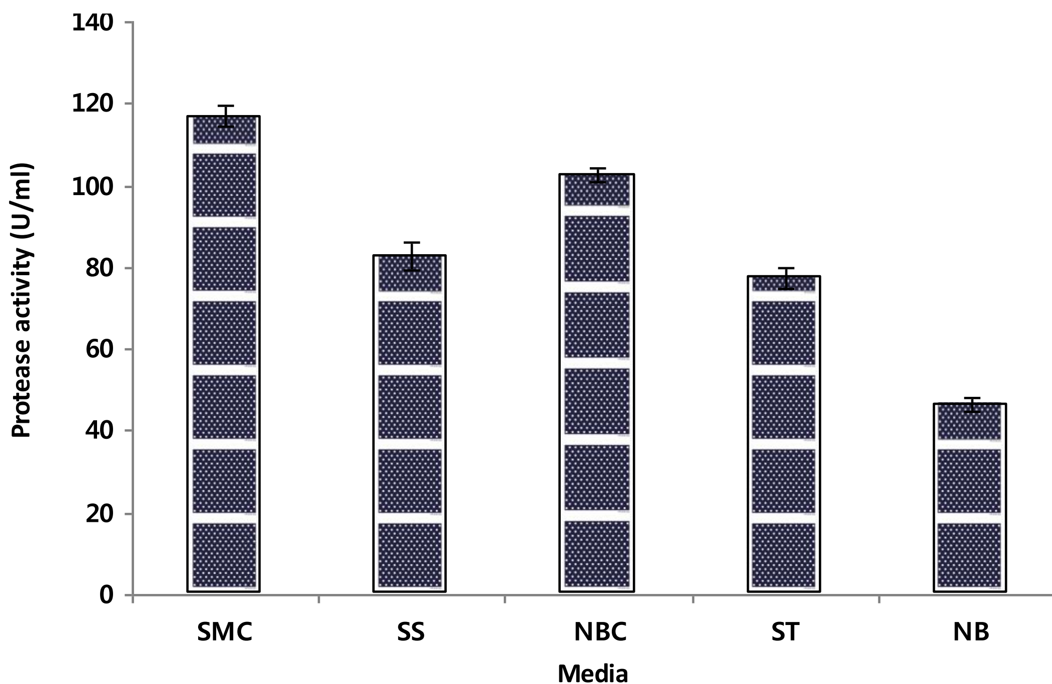

Figure 4. Effect of different media on protease production. Data represents the mean ± standard deviations of three replications.

U/ml) than that of under static culture conditions (61.17 ± 2.48 U/ml). The production of protease was found to decrease as the shaken speed increased up to 200 rpm. This may be correlated with breakdown of enzyme due to greater aeration and high shaking speed. The present finding is consistent with Razak et al. (1997) who observed the highest protease production at 150 rpm shaking speed for the *Bacillus* spp. On the other hand, Rahman et al. (2005) reported the highest alkaline protease production by *Pseudomonas aeruginosa* under

static conditions.

The effect of different inoculum sizes (1 to 10%) on protease production was studied and maximum protease activity (151.29 ± 2.85 U/ml) was observed at 1% inoculum size. Further increase in the inoculum size up to 10% had no influence on the enzyme production. High inoculum size could result in lack of oxygen and nutrient depletion in the media. This was confirmed by Haritha et al. (2012) who also observed reduction in protease production by 36.3 and 58.4% in the inoculum size at 8.0 and 10.0%, respectively due to lack of oxygen and nutrient depletion in the culture media. Similar reports on the maximum protease production under 1% inoculum size were observed by other researchers. Zambare et al. (2011) and Nilegaonkar et al. (2007) reported maximum protease production under 1% inoculum size by *P. aeruginosa* and *Bacillus cereus* respectively.

The cultivation volume of 100 ml medium in 500 ml capacity erlrnmeyer flask (1:5) was found to be the best volume for achieving high protease production (168.2 ± 4.58 U/ml). This may be due to high oxygen availability for the protease production. On the other hand, cultivation volume of 150 to 350 ml was found to decrease the production of protease drastically due to lack of oxygen. The present results are in agreement with Oh et al. (2000) who assessed the protease activity of *P. aeruginosa* K-187.

Effect of pH on activity and stability of protease

The results of the effect of pH on protease activity are shown in Figure 5a. The protease was active over the entire tested pH range from 5 to 11. The highest protease activity (100%) was found at pH 8. Enzyme activity decreased gradually under acidic conditions, showing approximately 87% of its original activity at pH 6 and 5. Furthermore, approximately 60% of relative activity was still detectable at pH 10 and 11 indicating that protease was active over a wide range of pH. These results are in accordance with previous reports which showed optimum pH of 8 for several bacterial strains such as *Bacillus licheniformis* Lbb-11(Olajuyigbe and Ajele, 2008), and *Pseudomanas aeruginosa* MCM B-327 (Zambare et al., 2011) that exhibited a maximum activity at pH 8.

The stability of the enzyme under different pH values was assayed by measuring the residual activity after overnight incubation at various pH (5 to 11). The optimum stability was shown to be in the pH 9 while protease retained more than 90% of its activity at pH 8, 10 and 11. According to the results, it is clear that protease of *B. stabilis* was stable over a wide basic pH range (8 to 11) and pH had very little effect on the stability of this enzyme. This highlights the suitability of this protease to use various industrial processes which are carried out at alkaline conditions. Many proteases are reported to exhibit stability in broad range of pH. Proteases from *Aeromonas veronii* PG01 (Divakar et al., 2010), and *Alcaligenes faecalis* (Thangam and Rajkumar, 2000) also showed stability over a wide basic pH range (8 to 11).

Effect of temperature on activity and stability of protease

Effect of temperature on protease activity and stability are shown in Figure 5b. The protease was active between 30 and 80°C with an optimum at 50°C. The activity was found to be increased rapidly after 40°C, however decreased after 50°C. The temperature stability showed that the enzyme retained almost 100% activity at room temperature (30°C) and at 40°C. The enzyme lost its original stability approximately by 50% at 60 to 80°C. There are many reports about temperature stability and activity of protease. Najafi et al. (2005) reported about protease from *Pseudomonas aeruginosa* PD100 with optimum activity and stability at 60 and 55°C, respectively.

Effect of metal ions and inhibitors on protease activity

As shown in Table 1, Ca^{2+} was found to increase protease activity by around 148% at the concentration of 10 mM. The protease activity was slightly enhanced by Mg^{2+} and Mn^{2+} (106 and 109%, respectively). The inhibitory effects were found in the presence of Zn^{2+}, Ni^{2+} and Sn^{2+} by 59, 47 and 67% respectively at the concentration of 10 mM. This finding explained that protease did not require the presence of Zn^{2+} ions to be active. The present findings are in agreement with that of Karbalaei-Heidari et al. (2007) who reported that Ca^{2+} Mg^{2+} and Mn^{2+} ions enhanced proteolytic activity, whereas Zn^{2+} and Ni^{2+} ions exhibited inhibitory effect on the proteolytic activity of zinc-metallo protease from halophilic bacterium *Salinivibrio* sp. strain AF-2004. The increased protease activity in the presence of Ca^{2+} may be attributed to the stabilization of enzyme rather than involving the catalytic reactions under excess Ca^{2+} concentrations. Similar reports on the effects of metal ion for other bacterial proteases have also been observed by researchers. In the presence of Zn^{2+}, a strong inhibitory effect on the protease activity was reported in *Bacillus megaterium* (Yossan et al., 2006), *Clostridium* sp. (Alam et al., 2005) and *Aeromonas veronii* PG01 (Divakar et al., 2010). The reason may be due to Zn^{2+} which usually serves as a co-factor of many enzymes which could be replaced by heavy metals, thereby making the enzymes inactive. Furthermore, excess Zn^{2+} is involved in the inhibition of several zinc peptidases (Windle et al., 1997). As shown in Table 1, no inhibition was observed in the presence of PMSF, suggesting that the enzyme is not a serine protease. The enzyme was inhibited by approximately 50% in the presence of EDTA, DTNB and

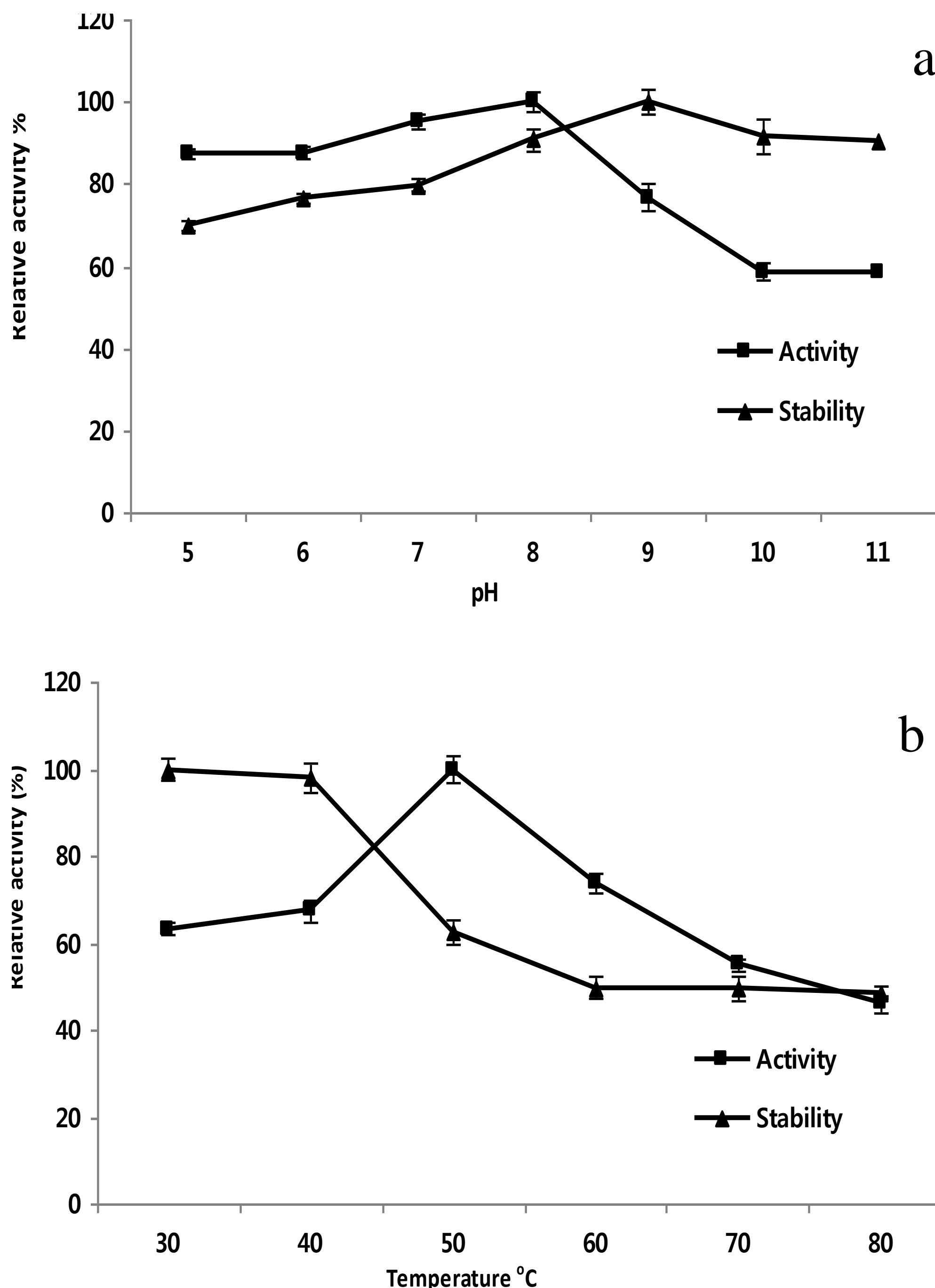

Figure 5. Effect of (a) pH and (b) temperature on protease activity and stability. Data represents the mean ± standard deviations of three replications.

β-ME. These results suggest that this protease could belong to the class of metalloproteases.

Substrate specificity and enzyme kinetics

Effect of different substrates on protease activity was assessed using different protein substrates such as casein, BSA, egg albumin and gelatin. The highest protease activity (151.29 ± 5.67 U/ml) was exhibited by casein, while the other substrates reported very low activities (37.8 ± 1.12, 46.3 ± 1.57 and 42.13 ± 3.24 U/ml, respectively for BSA, egg albumin and gelatin). Similarly, the highest protease activity towards casein was observed by other researchers for *Bacillus stearothermophillus* F1 (Rahman et al., 1994) and

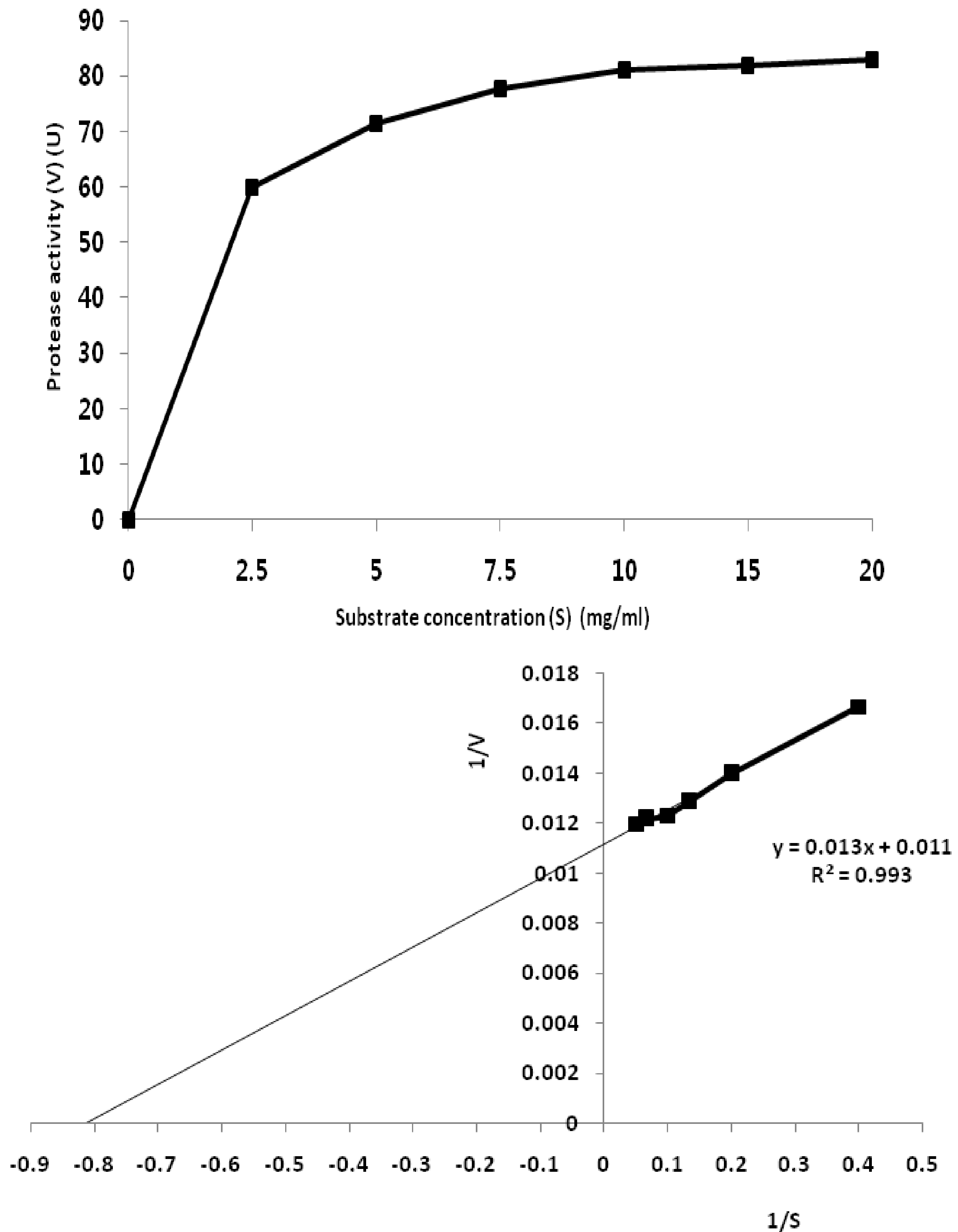

Figure 6. Lineweaver-Burk plots for *Burkholderia stabilis* protease under varying substrate concentrations (1-20 mg/ml) indicating the V_{max} and K_m values.

Bacillus subtillis PE1 (Adinarayana et al., 2003).

Optimum substrate concentration for maximum enzyme activity was determined in terms of V_{max} and K_m using casein as a substrate and data were interpreted from Lineweaver-Burk plots (Figure 6). Results reveal that protease from *B. stabilis* V_{max} and K_m was 89.28 U/ml and 0.82 mg/ml, respectively. Similar results were observed by Kalpana Devi et al. (2008) for *Aspergillus niger* with

Table 1. Effects of metal ions and inhibitors on protease activity.

Metal ion	Concentration (mM)	Relative activity (%)
Zn^{2+} ($ZnCl_2$)	1	63.72 ± 1.15
	10	59.53 ± 1.43
Ca^{2+} ($CaCl_2$)	1	103.71 ± 2.93
	10	148.14 ± 3.04
Mg^{2+} ($MgCl_2$)	1	103.72 ± 1.46
	10	106.51 ± 1.32
Mn^{2+} ($MnCl_2$)	1	103.72 ± 2.04
	10	109.54 ± 1.98
Ni^{2+} ($NiCl_2$)	1	58.76 ± 0.98
	10	47.34 ± 0.94
Sn^{2+} ($SnCl_2$)	1	69.31 ± 1.59
	10	67.21 ± 2.03
PMSF	1	91.32 ± 1.21
	10	96.67 ± 2.13
EDTA	1	57.93 ± 1.54
	10	52.93 ± 1.57
DTNB	1	40.33 ± 1.57
	10	37.11 ± 0.83
β-ME	1	46.35 ± 1.26
	10	38.21 ± 0.67

Data represents the mean ± standard deviations of three replications.

Table 2. Purification table for *B. stabilis* LMG14294 protease.

Purification step	Protease activity (U/ml)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture supernatant	117.3	86	1.36	100	1
Ammonium sulphate precipitation	74.97	3.8	19.73	63.91	14.51
Gel filtration chromatography	44.85	1.344	33.37	38.24	24.54

V_{max} and K_m values 85 U/ml and 0.8 mg/ml, respectively. Matta et al. (1994) also reported protease with lower K_m value with casein as substrate from *Bacillus alkalophilus*. According to these findings, it is clear that *B. stabilis* indicates lower affinity of the enzyme towards casein.

Protease purification and molecular weight determination

Purification of the extracellular protease from *B. stabilis* was carried out by combination of ammonium sulphate precipitation (50, 60 and 70%) and gel filtration chromatography procedures. The results of the purification procedure are summarized in Table 2. After ammonium sulphate precipitation, protease attained 14.46 purification fold and 63.91% yield. The precipitated fraction was subjected to gel filtration on a Sephadex G-300 column after dialysis. This yielded one peak for protease activity having 38.24% yield. Apparent molecular mass of the protease was about 47 kDa (Figure 7). The molecular mass of *B. stabilis* protease was comparable with most of

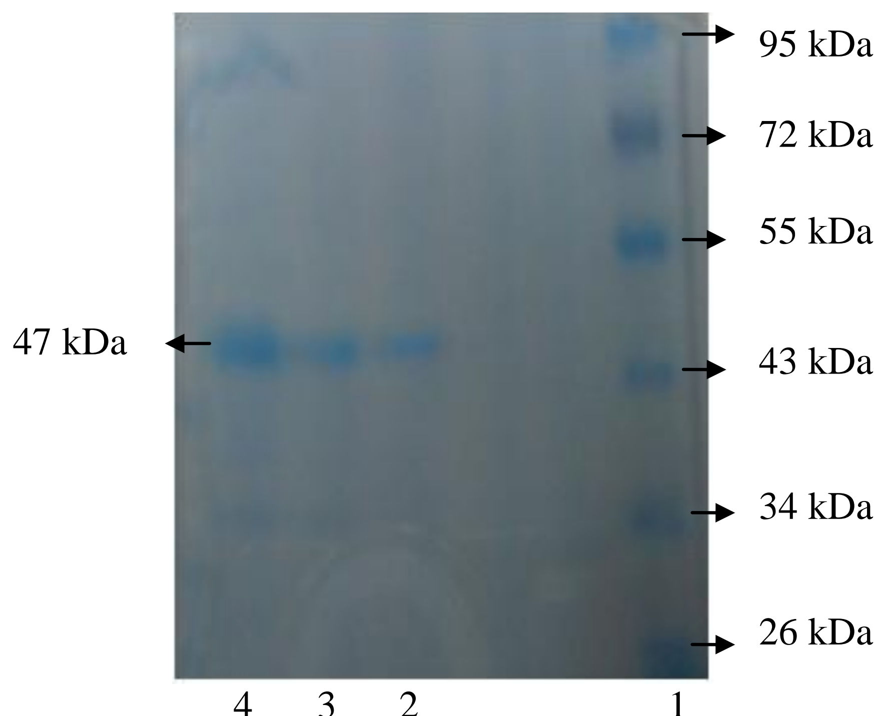

Figure 7. SDS PAGE analysis of purified protease. Lane 1, Molecular weight marker; lane 2, crude protease; lane 3, $NH_4(SO_4)_2$ precipitation; lane 4, purified protease.

the other bacterial strain proteases. Similar molecular masses were reported by Zibaee and Bandani (2009) for *Beauveria bassiana* (47 kDa) and Nileganokar et al. (2007) for *Bacillus cereus* (45 kDa).

It can be concluded that the isolated strain of *B. stabilis* was found to be able to secrete proteolytic enzyme. Due to the possession of desirable properties such as activity and stability under wide range of pH and temperature conditions, the presence of different metal ions indicate that the proteases from *B. stabilis* can be supposed to be suitable for commercial applications.

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